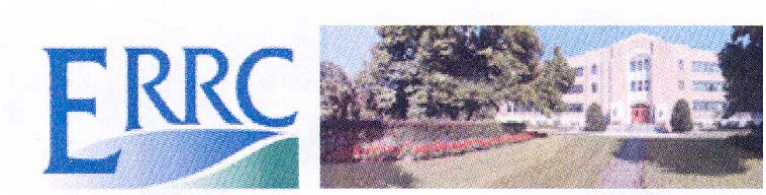

EASTERN REGIONAL RESEARCH CENTER
AGRICULTURAL RESEARCH SERVICE
UNITED STATES DEPARTMENT OF AGRICULTURE
600 E. MERMAID LANE
WYNDMOOR, PA 19038
(215) 233-6400

Title: Reduction of the Native Microflora on Alfalfa Sprouts During Propagation by Addition of Antimicrobial Compounds to the Irrigation Water

Author(s): W.F. Fett,

Citation: International Journal of Food Microbiology (2002) 72:13-18

Number: 7062

Reduction of the native microflora on alfalfa sprouts during propagation by addition of antimicrobial compounds to the irrigation water ☆

William F. Fett *

Plant Science and Technology Research Unit, Eastern Regional Research Center, Agricultural Research Service, US Department of Agriculture, 600 E. Mermaid Lane, Wyndmoor, PA 19038, USA

Received 12 February 2001; accepted 30 June 2001

Abstract

Alfalfa and other types of sprouts are known to harbor large populations of native microorganisms. As some of these microbes may be causes of reduced shelf life of the product (plant pathogens and other spoilage organisms) and sprouts may, on occasion, harbor bacteria pathogenic towards humans, the addition of antimicrobial compounds to the irrigation water may be warranted. In this study, we tested the efficacy of several antimicrobial compounds for reducing the native microbial populations on alfalfa sprouts during propagation. These compounds included H_2O_2, peroxyacetic acid + hydrogen peroxide (Tsunami 100™), acidified $NaClO_2$, $NaClO_2$ (Aquatize™), EDTA, Na_3PO_4 and NaOCl. When added to the irrigation water at various concentrations, none of the antimicrobial compounds reduced the levels of any class of native microflora by more than 1 $\log_{10}$ without evidence of phytotoxicity. Published by Elsevier Science B.V.

Keywords: Bacteria; Sprout; Antimicrobial; Alfalfa; Yeast, mold

1. Introduction

A wide variety of fresh, raw green sprouts are consumed usually as components of salads and sandwiches, with alfalfa sprouts being consumed in the greatest quantity in the USA. Unfortunately, in the USA and in several other countries, there have been several recent outbreaks of illness caused by sprouts contaminated with *Salmonella* or *Escherichia coli* O157:H7 (NACMCF, 1999; Taormina et al., 1999). The primary source of these bacterial contaminants is generally believed to be the seed. Seed is obtained from plants grown in the open field similar to other agricultural crops, usually with no special precautions for seed that will eventually be used for sprout production. In 1998, the US National Advisory Committee on Microbiological Criteria for Foods identified sprouts as a special food safety problem (NACMCF, 1999) due to the ability of bacterial human pathogens to grow from low numbers on the seed to very high numbers on the sprouts due to favorable temperatures, moisture and nutrient availability during propagation. On July 9, 1999, the USA Food and Drug

☆ Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture above others of a similar nature not mentioned.

* Corresponding author. Tel.: +1-215-233-6418; fax: +1-215-233-6559.

E-mail address: wfett@arserrc.gov (W.F. Fett).

Administration (FDA) released a consumer advisory warning the consuming public not to eat raw sprouts if they wished to reduce their chances of contracting food-borne illness, http://vm.cfsan.fda.gov/~lrd/hhssprts.html).

Several intervention steps have the potential for reducing contamination with bacterial human pathogens at sprouting facilities. The first is seed treatment with antimicrobial compounds. In two recent guidance documents, the FDA recommended treatment of sprouting seed with 20,000 mg/l of free chlorine from calcium hypochlorite or an equivalent antimicrobial treatment along with testing of the spent irrigation water for *Salmonella* and *E. coli* O157:H7 (http://vm.cfsan.fda.gov/~dms/sprougd1.html and, http://vm.cfsan.fda.gov/~dms/sprougd2.html). Currently, the only seed treatment approved by the US EPA is the use of chlorine up to 20,000 mg/l. Two possible additional intervention steps are the addition of antimicrobial compounds to the irrigation water and the use of an antimicrobial intervention step postharvest.

In this study, we tested the effect of the addition of several compounds with known antimicrobial activity to the irrigation water on the populations of the native microorganisms. In addition to being of value for the production of safer sprouts, this intervention strategy also has the potential for reducing postharvest spoilage leading to a longer product shelf life.

2. Material and methods

Alfalfa seed was obtained from Caudill Seed, Louisville, KY, USA. Seed was either untreated or was treated with 3800 (from NaOCl) or 13,000 or 20,000 [from $Ca(OCl)_2$] mg/l of free chlorine. To prepare the NaOCl-containing solutions, 40 ml of a commercial bleach preparation (Clorox™, Oakland, CA, 5.25% NaOCl) was added to 460 ml of sterile 50 mM potassium phosphate buffer (KP buffer), pH 6.8. To prepare the 13,000 and 20,000 mg/l free chlorine solutions, 10 or 15 g of $Ca(OCl)_2$ granules (Aldrich Chemical, Milwaukee, WI, 65% available chlorine) were added to 500 ml of 500 mM KP buffer, respectively, and the solutions stirred for 20 min (final pH = 7.0). Final concentrations of free chlorine in the solutions were determined by use of a colorimetric assay (Reflectoquant free chlorine strips and reader, EM Science, Gibbstown, NJ). Seeds (30 g) were stirred in the sanitizer solutions (200 ml) by hand for 10 min followed by two sequential rinses with sterile tap water (200 ml per rinse). Microbial populations on alfalfa seed were determined before and after treatment with free chlorine by pummeling 25 g samples of seed in 50 ml of 0.1% sterile peptone-water (PW) for 30 s in a model 400 stomacher (Seward, London, UK) set at medium speed. Decimal dilutions were prepared in sterile PW. One milliliter of each dilution was plated in duplicate onto Petrifilm™ *E. coli*/coliforms, general aerobes and yeast and mold plates (3M, St. Paul, MN) and the inoculated plates incubated at 35 °C, 30 °C or room temperature, respectively. Final counts of generic *E. coli*, coliforms and mesophilic aerobes on the plates were determined after 48 h of incubation, while final yeast and mold counts were determined after 5 days of incubation. The experiment was done two times.

Before propagation, treated or untreated seed was soaked for 4 h in sterile tap water and placed into small plastic trays (260 × 160 × 64 mm; 10 g seed/tray) with three holes drilled at one end to allow drainage of irrigation water. The small trays were then placed into much larger trays contained in a sprouting chamber kept at room temperature with automatic overhead spray irrigation. Irrigation water with or without chemical additives was fed into the system from a large carboy (25 l) and sprouts were irrigated for 1 min out of every 30 min. Fresh solutions were added to the carboy each morning.

The following irrigation solutions containing antimicrobial compounds were prepared in tap water in 20 l volumes: (1) Hydrogen peroxide (30%, Fisher Scientific, Pittsburgh, PA) at 200 and 1000 mg/l. The pH was either left unadjusted (final pH = 7.2) or the pH was adjusted to a final pH of 5.0 by addition of 1.75 M acetic acid (36 ml) (200 mg/l solutions only) (Mallinckrodt Baker, Paris, KY). Peroxide concentrations and stability over a period of 24 h were confirmed by use of Reflectoquant peroxide test strips and reader (EM Science). (2) Tsunami 100™ (Ecolab, St. Paul, MN), a mixture of peroxyacetic acid (15%) and hydrogen peroxide (11%). Irrigation solutions were prepared in tap water containing 20, 40 and 80 ppm peroxyacetic acid, final pHs of 6.6, 6.1 and 5.2, respectively. Peroxide concentrations and stability over a period of 24 h were confirmed as stated above. (3) Acidified

$NaClO_2$ (Alcide, Redmond, WA), provided by the manufacturer as a 12% $NaClO_2$ concentrate, pH 10.7. The following formulations containing this product were tested: 12, 30 and 60 mg/l of $NaClO_2$ with the final pH adjusted to 5.0 by addition of 1.75 M acetic acid (40 to 60 ml). (4) Sodium chlorite (Aquatize™, 3.67% $NaClO_2$, pH 11.6, Bioxy, Raleigh, NC). The concentrate was tested at 18 and 36 mg/l, final pH = 7.6. (5) Acetic acid at 0.088 M (0.05%, v/v), final pH = 3.5. (6) Trisodium phosphate (food grade, Rhone-Poulenc, Cranbury, NJ) at 0.3% (w/v), final pH = 11.0. (7) EDTA (disodium dihydrate, Sigma, St. Louis, MO) at 50, 100, 150, 200 and 400 mg/l (final pH = 6.5–6.8). (8) NaOCl (10–13% available chlorine, Sigma) at 100 and 200 mg/l of free chlorine as determined using Reflectoquant test strips for free chlorine and reader, final pH adjusted to 6.8 with H_2SO_4. The dilutions of the chlorine-containing solutions required before use of the test strips were prepared in glass beakers pretreated with commercial bleach to remove any chlorine demand. Beakers were soaked in a 1:1000 dilution of commercial bleach for 1.5 h, rinsed well with highly purified water (18.2 MΩ cm resistivity) and then dried in an oven. The tap water had a pH of 7.0–7.2 and contained approximately 120 mg/l total hardness as $CaCO_3$ as determined with water quality test strips for hardness (Hach Company).

After 4 days, two 25-g samples of the sprouts were removed from each of the three trays for microbiological analysis. One sample from each tray was placed into a filter stomacher bag (Daigger Scientific, Vernon Hills, IL) containing 100 ml of sterile 100 mM potassium phosphate buffer, pH 6.8 (KP buffer) and the bag contents were gently massaged by hand for 2 min. The second sample from each tray was placed into a sterile blender receptacle, 100 ml of KP buffer added and homogenized for 30 s using a commercial blender. Serial decimal dilutions were prepared in sterile 0.1% peptone-water. One milliliter of the dilution was placed onto Petrifilm general aerobe plates, *E. coli*/coliform plates, *Enterobacteriaceae* plates and yeast and mold plates (3M) with two plates inoculated per dilution. The inoculated general aerobe plates were incubated at 30 °C, the *E. coli*/coliform and *Enterobacteriaceae* plates at 35 °C and the yeast and mold plates at room temperature. Final readings were made after 48 h except for yeast and molds (4 days).

When antimicrobials were tested in more than a single experiment, the means of the data from each of the three individual trays per experiment were used to calculate the standard error of the means.

Phytotoxicity was estimated by visual inspection with the naked eye.

3. Results and discussion

Initial populations of mesophilic aerobes, yeasts and molds and coliforms on the untreated seed were 4.08 ± 0.02 $\log_{10}$ cfu/g, 3.07 ± 0.03 $\log_{10}$ cfu/g and 1.22 ± 0.32 $\log_{10}$ cfu/g, respectively. No generic *E. coli* was detected. Treatment of the seed with 3800 mg/l of free chlorine from NaOCl resulted in a reduction of the microbial populations to 20 or less cfu/g for total mesophilic aerobes and less than 1 cfu/g (the limit of detection) for yeasts and molds and coliforms (results not shown). Populations of all classes of microbes were below the limits of detection after treatment of seed with the higher levels of free chlorine.

The populations of mesophilic aerobes (approximately 8 $\log_{10}$ cfu/g), coliforms (approximately 7 $\log_{10}$ cfu/g) and yeast and molds (3 to 4 $\log_{10}$ cfu/g) determined in this study for sprouts grown without any antimicrobials additives to the irrigation water were similar to those reported previously for various types of sprouts (Andrews et al., 1982; Sly and Ross, 1982; Splittstoesser et al., 1983; Patterson and Woodburn, 1980; Prokopowich and Blank, 1990). Treatment of the seed with various concentrations of chlorine before sprouting did not affect the final microbial populations on the harvested alfalfa sprouts (Table 1).

In preliminary experiments, the stability of various irrigation solutions containing antimicrobials maintained at room temperature (20–22 °C) for 24 h was examined. A 400-mg/l solution of free chlorine prepared from NaOCl and adjusted to a pH of 6.2 with citric acid (0.425 g/l) contained only 21–25 mg/l of free chlorine after 24 h. When citric acid was used to adjust the pH to 7.0 (0.25 g/l), approximately 40% of the initial level of free chlorine remained. The concentration of free chlorine in the solution remained unchanged when adjusted to a pH of 8.0 using 0.04 g/l of citric acid. When the pH was adjusted with mineral acid (H_2SO_4), concentrations of solutions at all three

Table 1
Populations of native microorganisms recovered from alfalfa sprouts irrigated with antimicrobial chemicals

Seed treatment	Irrigation chemical	No. of Experiments[a]	Mixing[b]	Log_{10} cfu/g				
				Mesophilic aerobic bacteria	Coliforms	Enterobacteriaceae	Yeast and molds	Phytotoxicity
None	tap water	2	AH	8.42 ± 0.07[c]	7.77 ± 0.34	ND	4.13 ± 0.01	–
			H	8.63 ± 0.02	7.97 ± 0.40	ND	4.40 ± 0.3	
Chlorine, 3800 mg/l	tap water	2	AH	8.31 ± 0.09	7.48 ± 0.10	ND	3.80 ± 0.43	–
			H	8.52 ± 0.03	7.52 ± 0.20	ND	3.87 ± 0.41	
Chlorine, 13,000 mg/l	tap water	2	AH	8.46 ± 0.12	7.16 ± 0.25	7.53 ± 0.15	3.07 ± 0.47	–
			H	8.59 ± 0.17	7.32 ± 0.19	7.60 ± 0.38	3.59 ± 0.42	
Chlorine, 20,000 mg/l	tap water	2	AH	8.59 ± 0.10	7.03 ± 0.18	7.35 ± 0.13	4.27 ± 0.34	–
			H	8.75 ± 0.03	7.37 ± 0.17	7.67 ± 0.14	4.51 ± 0.43	
Chlorine, 3800 mg/l	H_2O_2 pH 7.2, 200 mg/l	1	AH	8.56	7.55	ND[d]	3.94	–
			H	8.81	7.45	ND	4.02	
Chlorine, 13,000 mg/l	H_2O_2 pH 7.2, 20 mg/l	1	AH	8.53	7.39	ND	3.02	–
			H	8.80	7.29	ND	3.30	
Chlorine, 13,000 mg/l	H_2O_2 pH 5.0, 200 mg/l	3	AH	8.33 ± 0.22	7.50 ± 0.26	7.96 (No. = 1)[a]	4.20 ± 0.83	–
			H	8.41 ± 0.16	7.19 ± 0.29	7.56 (No. = 1)[a]	4.48 ± 0.53	
Chlorine, 13,000 mg/l	Tsunami 100, 20 mg/l	1	AH	8.38	8.00	ND	4.69	–
			H	8.42	8.02	ND	4.59	
Chlorine, 13,000 mg/l	Tsunami 100, 40 mg/l	1	AH	8.54	6.93	ND	5.26	–
			H	8.61	6.64	ND	5.18	
Chlorine, 20,000 mg/l	$NaClO_2$ pH 5, 12 mg/l	1	AH	8.54	6.40	6.59	5.26	–
			H	8.80	6.18	6.78	5.31	
Chlorine, 20,000 mg/l	$NaClO_2$ pH 5, 30 mg/l	1	AH	8.67	7.38	7.44	>5.60	–
			H	8.72	7.03	7.17	>5.60	
Chlorine, 20,000 mg/l	Na_3PO_4, 3000 mg/l	1	AH	8.82	7.62	7.77	4.78	+
			H	9.29	7.64	7.79	4.90	
Chlorine, 20,000 mg/l	Aquatize™, 18 mg/l	1	AH	8.40	6.99	7.33	5.12	–
			H	8.76	7.40	7.56	5.15	
	36 mg/l	1	AH	8.67	6.85	7.18	4.97	+
			H	8.81	6.39	7.03	5.20	
Chlorine, 13,000 mg/l	EDTA, 50 mg/l	1	AH	9.14	7.85	8.16	>5.60	–
			H	9.12	7.73	7.86	>5.60	
	100 mg/l	1	AH	8.41	7.16	7.22	5.18	–
			H	8.71	7.31	7.37	5.22	
	150 mg/l	1	AH	8.53	6.51	7.49	5.15	–
			H	8.84	6.72	7.62	5.17	
	200 mg/l	1	AH	9.22	6.67	6.82	4.76	–
			H	8.84	6.94	7.57	4.77	
	400 mg/l	1	AH	8.70	7.37	7.66	4.86	–
			H	8.88	7.18	7.89	5.56	
Chlorine, 13,000 mg/l	NaOCl, pH 6.8, 100 mg/l	1	AH	8.16	7.05	7.40	2.64	+
			H	8.13	6.95	7.26	2.94	
	200 mg/l	2	AH	8.19 ± 0.18	7.33 ± 0.25	7.56 (No. = 1)[a]	4.40 ± 0.21	+
			H	8.32 ± 0.07	7.34 ± 0.05	7.49 (No. = 1)[a]	4.50 ± 0.21	

[a] Number of experiments (three repetitions per experiment except where noted).
[b] AH = agitated by hand, H = homogenized.
[c] Values are means ± standard error.
[d] ND = not detected.

initial pHs remained constant over this time period. Hydrogen peroxide at acidic as well as neutral pH was tested as it is reported to have greater antibacterial activity at pH 5 than at 7 (Cords and Dychdala, 1993). The concentration of peroxide-containing solutions (Tsunami 100 and H_2O_2) also remained constant.

None of the antimicrobial compounds automatically applied for 1 min every 30 min at the concentrations tested were able to significantly reduce the populations of the native microflora on alfalfa sprouts and several were phytotoxic based on visual inspection with the naked eye alone (Table 1). Additional irrigation solutions not shown in Table 1, which proved to be phytotoxic were acetate buffer (20 mM, pH 5.0), acetic acid (0.088 M, pH 3.5), acidified $NaClO_2$ (60 mg/l, pH 5), H_2O_2 (1000 mg/l, pH 7), peroxyacetic acid (80 mg/l, pH 5.2), and Na_3PO_4 (0.3%, pH 11). Symptoms of phytotoxicity ranged from slight curling of fully-grown sprouts to almost complete inhibition of germination and growth. None of the affected sprouts were deemed to be marketable. When determined, the levels of the native microflora on the damaged sprouts were also not greatly reduced (Table 1).

In general, two to three times higher plate counts were recovered on the various media when homogenization of the sprouts was done rather than massaging by hand (Table 1). This difference may be attributable to the presence of biofilms on the sprouts as previously demonstrated in our laboratory by scanning electron microscopy (Fett, 2000). Also, based on the results of the present study and a previous study (Fett, 2000), homogenization does not appear to release any significant amount of antimicrobial compounds from the plant tissues which would lead to lowering of plate counts. The presence of biofilms may account, at least in part, for the inability of the various antimicrobials to significantly reduce the levels of the natural microflora. Bacteria in biofilms are 500-fold or more resistant to the action of antimicrobials than planktonic forms (Costerton et al., 1995). In addition, using immunofluorescence and immunoelectron microscopy techniques as well as direct isolation from surface-sterilized radish sprout tissues, Itoh et al. (1998) demonstrated that *E. coli* O157:H7 may be able to enter into the internal tissues of sprouts where they would be protected from the effects on nonsystemic antimicrobials sprays.

In studies with mung beans, Splittstoesser et al. (1983) found that application of water rinses containing 50–100 mg/l of chlorine during propagation reduced the total aerobe and coliform counts by less than 1 log. Daily irrigation of alfalfa sprouts with 100 mg/l chlorine dioxide did not reduce populations of total mesophilic aerobes over an 8-day growing period (Castro-Rosas and Escartin, 1999). In the same study, similar experiments using seed grown from seed inoculated with *Vibrio cholerae* resulted in a maximum reduction (2 $\log_{10}$) in the population of the pathogen at day 3, but a significant population (>2 $\log_{10}$ cfu/g) of the pathogen remained. Taormina and Beuchat (1999) tested a variety of antimicrobials compounds for their ability to control growth of *E. coli* O157:H7 on growing alfalfa sprouts including free chlorine [200 and 2000 mg/l from NaOCl or $Ca(OCl)_2$], 100, 500 and 1200 mg/l of acidified $NaClO_2$, 40 and 80 mg/l of peroxyacetic acid, 10,000 mg/l of Na_3PO_4 and 10,000 mg/l of H_2O_2. Only acidified $NaClO_2$ at 1200 mg/l was able to control the growth of the pathogen on the growing sprouts, but it did not eliminate or significantly reduce the populations from those present on the laboratory-inoculated seed before sprouting. In those studies, sprouts were grown in boxes in the laboratory and were sprayed once per day over the 4-day growing period. Five minutes after treatments, the treated sprouts were rinsed by spraying with sterile tap water. The most promising report to date was that of Naito and Shiga (1989). They found that treatment of growing alfalfa and bean sprouts with a combination of 0.2 mg/l ozone in air and 0.3–0.5 mg/l ozonated water led to a reduction of 2 to 3 $\log_{10}$ in total microbial counts and a reduction of 3 to 4 $\log_{10}$ in total coliforms after 7 days.

In conclusion, the native microbial population on growing alfalfa sprouts could not be reduced by the addition of various antimicrobials to the sprout irrigation water. In addition, several of the tested solutions were phytotoxic. Due to the fragile nature of the growing sprouts, as well as the fact that much of the native microflora is present in the form of biofilms, significant reduction of the microflora population using this intervention strategy may not be possible. Addition of antimicrobial compounds to irrigation water would also preclude the testing of spent irrigation water for bacterial pathogens as an indicator of sprout contamination as recommended by the FDA. To help insure the microbial safety of sprouts as well as increase shelf life, treatment of sprouting seed and postharvest treatments of sprouts by chemical, physical and/or biological means appear to be more viable alternatives.

Acknowledgements

The author wishes to thank Mr. James Butterfield for his expert technical assistance.

References

Andrews, W.H., Mislivec, P.B., Wilson, C.R., Bruce, V.R., Poelma, P.L., Gibson, R., Trucksess, M.W., Young, K., 1982. Microbial hazards associated with bean sprouting. J. Assoc. Off. Anal. Chem. 65 (2), 241–248.

Castro-Rosas, J., Escartin, E.F., 1999. Incidence and germicide sensitivity of *Salmonella typhi* and *Vibrio cholerae* O1 in alfalfa sprouts. J. Food Saf. 19, 137–146.

Cords, B.R., Dychdala, G.R., 1993. Sanitizers: halogens, surface-active agents, and peroxides. In: Davidson, P.M., Branen, A.L. (Eds.), Antimicrobials in Foods, 2nd edn. Marcel Dekker, New York, pp. 469–537.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappin-Scott, H.M., 1995. Microbial biofilms. Annu. Rev. Microbiol. 49, 711–745.

Fett, W.F., 2000. Naturally occurring biofilms on alfalfa and other types of sprouts. J. Food Prot. 63 (5), 625–632.

Itoh, Y., Sugita-Konishi, Y., Kasuga, F., Iwaki, M., Hara-Kudo, Y., Saito, N., Noguchi, Y., Konuma, H., Kumagai, S., 1998. Enterohemorrhagic *Escherichia coli* O157:H7 present in radish sprouts. Appl. Environ. Microbiol. 64, 1532–1535.

Naito, S., Shiga, I., 1989. Effect of ozone treatment on elongation of hypocotyl and microbial counts on bean sprouts. J. Jpn. Soc. Food Sci. Technol. 36 (3), 181–188.

National Advisory Committee on Microbiological Criteria for Foods, 1999. Microbiological safety evaluations and recommendations on sprouted seeds. Int. J. Food Microbiol. 52, 123–153.

Patterson, J.E., Woodburn, M.J., 1980. *Klebsiella* and other bacteria on alfalfa and bean sprouts at the retail level. J. Food Sci. 45, 492–495.

Prokopowich, D., Blank, G., 1990. Microbiological evaluation of vegetable sprouts and seeds. J. Food Prot. 54 (7), 560–562.

Sly, T., Ross, E., 1982. Chinese foods: relationship between hygiene and bacterial flora. J. Food Prot. 45 (2), 115–118.

Splittstoesser, D.F., Queale, D.T., Andaloro, B.W., 1983. The microbiology of vegetable sprouts during commercial production. J. Food Saf. 5, 79–86.

Taormina, P.J., Beuchat, L.R., 1999. Behavior of enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa sprouts during the sprouting process as influenced by treatments with various chemicals. J. Food Prot. 62 (8), 850–856.

Taormina, P.J., Beuchat, L.R., Slutsker, L., 1999. Infections associated with eating seed sprouts: an international concern. Emerging Infect. Dis. 5, 626–634.